ORIGINAL RESEARCH

# Age-Dependent Impairment in Endothelial Function and Arterial Stiffness in Former High Class Male Athletes Is No Different to That in Men With No History of Physical Training

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***Background*—**Physical activity is generally considered to exert positive effects on the cardiovascular system in humans. However, surprisingly little is known about the delayed effect of professional physical training performed at a young age on endothelial function and arterial stiffness in aging athletes. The present study aimed to assess the impact of long-lasting professional physical training (endurance and sprint) performed at a young age on the endothelial function and arterial stiffness reported in older age in relation to glycocalyx injury, prostacyclin and nitric oxide production, inflammation, basal blood lipid profile, and glucose homeostasis.

***Methods and Results*—**This study involved 94 male subjects with varied training backgrounds, including young athletes (mean age ~25 years), older former high class athletes (mean age ~60 years), and aged-matched untrained control groups. Aging increased arterial stiffness, as reflected by an enhancement in pulse wave velocity, augmentation index, and stiffness index ($P<10^{-4}$), as well as decreased endothelial function, as judged by the attenuation of flow-mediated vasodilation (FMD) in the brachial artery ($P=0.03$). Surprisingly, no effect of the training performed at a young age on endothelial function and arterial stiffness was observed in the former athletes. Moreover, no effect of training performed at a young age ($P>0.05$) on blood lipid profile, markers of inflammation, and glycocalyx shedding were observed in the former athletes.

***Conclusions*—**Our study clearly shows that aging, but not physical training history, represents the main contributing factor responsible for decline in endothelial function and increase in arterial stiffness in former athletes. **(*J Am Heart Assoc*. 2019;8: e012670. DOI: 10.1161/JAHA.119.012670.)**

**Key Words:** aging • arterial stiffness • cardiovascular disease risk factors • flow-induced dilation



Age is an independent risk factor of cardiovascular disease (CVD), given that more than 90% of all CVD (hypertension, coronary and peripheral artery disease, and stroke) occurs at ages above 40.[1,2] An increased risk of CVD with aging is mechanistically linked to an impairment of endothelial function and an increased stiffness of large elastic conduit arteries. In fact, endothelial dysfunction and increased arterial stiffness have a prognostic significance in age-dependent increase in CVD.[2,3] Age-dependent endothelial dysfunction is mainly linked to a decrease in nitric oxide ($NO^{\bullet}$) bioavailability and related to an enhancement of oxidative stress accompanied by chronic low-grade inflammation occurring with aging.[3] The lowering of $NO^{\bullet}$ bioavailability with age leads to proinflammatory, proliferative, hypercoagulative, and vasoconstrictive states in vessels that may contribute to an increased risk of CVD.[4,5] Interestingly, a decrease in $NO^{\bullet}$ bioavailability has been found to be implicated in an enhancement of vascular fibrosis and arterial stiffness.[6,7]

On the other hand, it has been well documented that regular aerobic physical activity reduces cardiovascular risk in middle-aged and older adults.[2] This effect might be partly explained by a training-induced reduction of cardiovascular risk factors, such as hyperlipidemia, hypertension, and insulin resistance, as well as by an improvement of $NO^{\bullet}$-dependent endothelial function and an attenuation of arterial stiffening.[8,9] Accordingly, a very strong evidence concerning the

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## Clinical Perspective

### What Is New?

- Former athletes at an older age display a comparable age-dependent decline in endothelial function and age-dependent increase in arterial stiffness to individuals with no history of physical training at a young age.
- This study shows that an age-related decline in endothelial function and an increase in arterial stiffness in former aged athletes are not dependent upon history of training performed at a young age.

### What Are the Clinical Implications?

- Professional sprint or endurance training performed at a young age is not harmful to endothelial function and arterial stiffness in young athletes.
- The potential beneficial effects of physical training performed at a young age disappear at the course of aging.

positive impact of physical activity on CVD risk factors has been pointed out by Fiuza-Luces et al,[9] underlying that in the Hadza people of Tanzania, who spend 950 minutes each week on moderate-to-vigorous physical activity (ie, 6-fold greater than 150 minutes per week—the minimum of physical activity recommended by the World Health Organization), no cardiovascular risk factors are present.[9] Those results confirm the suggestion that the impact of physical activity on cardiovascular health is "dose" dependent.[10]

Surprisingly little is known about the effect of professional long-lasting physical training performed at a young age on endothelial function and arterial stiffness in former athletes in older age. In the present study, we hypothesized that age-dependent endothelial dysfunction and increased arterial stiffness, as the strong predictors of CVD, are delayed in former athletes (endurance trained and sprinters) when compared with age-matched controls. We tested this hypothesis and assessed the impact of aging and physical training history on endothelial function and arterial stiffness in a group of men in relation to nitric oxide and prostacyclin production, markers of endothelial glycocalyx integrity, signs of systemic inflammation, blood lipid profile, and glucose homeostasis.

Endothelial function in the present study was evaluated by brachial flow-mediated dilation (FMD). Arterial stiffness of central elastic arteries was determined by central augmentation index (AI), whereas peripheral arterial stiffness was determined by carotid-radial pulse wave velocity (PWV) measurements and stiffness index (SI) calculation.[11] Moreover, additional CVD risk factors, such as central systolic blood pressure (cSBP), central pulse pressure (cPP), and carotid intima-media thickness (cIMT; as an index of subclinical atherosclerosis), were determined.

## Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Subjects

Ninety-four male subjects participated in this study: 47 young men (Y group, <45 years old; min–max, 18–43) and 47 older men (O group, >45 years old; min–max, 48–75). The group of young men (Y) consisted of 15 endurance-trained young athletes (Y-End, n=15), 15 sprint-trained young athletes (Y-Sp, n=15), and 17 untrained young controls (Y-Con, n=17). The group of older men (O) consisted of 16 former endurance-trained athletes (O-End, n=16), 15 former sprint-trained athletes (O-Sp, n=15), and 16 untrained older controls (O-Con, n=16). Physical training background and basic anthropometric data for all studied groups are given in Table 1.

All 4 groups of athletes (Y-End, Y-Sp, O-End, and O-Sp) consisted of individuals who currently (Y-End and Y-Sp) or previously (O-End and O-Sp) conducted several years of professional physical training (sprint or endurance) and successfully competed in their sporting events at a national and international level, including country championships, world championships, and the Olympics. The young athletes (Y-End and Y-Sp) at the time of study performed high-intensity training (≈790 versus 800 minutes per week, respectively, for Y-End and Y-Sp). The former athletes (O-End and O-Sp) at young age performed high-intensity professional training (≈1130 versus 730 minutes per week, respectively, for O-End and O-Sp), which was then followed by several years (from 15 to 44 years versus from 16 to 44 years, respectively, for O-End and O-SP) of low-to-average amount of physical activity per week (∼120 minutes per week; Table 1). Two untrained groups of subjects (both young and older) served as age-matched controls (Y-Con and O-Con).

The subjects that were recruited to this study had no history of serious metabolic, cardiovascular or endocrine disease (or any other known medical condition); however, some of the older subjects presented stage 1 hypertension and were taking blood pressure therapy (monotherapy, ie, 8 men from O-Con, 1 from O-End, and 1 from the O-Sp group). Moreover, additionally to blood pressure therapy, 2 subjects from O-Con group used statins. None of the subjects smoked or had any other addiction (eg, alcohol or drugs).

## Study Design

The study started with a medical health examination, basic anthropometric measurements, physical activity, and training background questionnaire. Based on this information, the 6 groups of subjects were identified and a comprehensive blood

**Table 1.** Basic Characteristics of the Studied Group of Men

| | Y-Con (n=17) | Y-End (n=15) | Y-Sp (n=15) | O-Con (n=16) | O-End (n=16) | O-Sp (n=15) |
|---|---|---|---|---|---|---|
| Age, y | 22.0±2.3 | 26.8±8.1 | 23.2±2.7 | 60.6±6.7 | 60.4±8.2 | 59.1±6.2 |
| Height, cm | 178±5.8 | 180±6.0 | 184±6.4 | 174±5.9 | 175±5.1 | 181±7.5 |
| Body mass, kg | 72.6±5.2 | 70.9±4.9 | 77.4±6.7† | 79.7±10.0* | 78.1±9.3* | 87.8±11.1*,† |
| BMI, kg/m$^2$ | 23.1±1.5 | 21.8±0.9 | 22.8±1.2 | 26.3±2.5* | 25.7±3.5* | 26.8±3.3* |
| Body fat, % | 15.6±2.5 | 13.1±1.8 | 13.2±1.5 | 26.1±3.9* | 24.2±6.5* | 24.8±6.5* |
| Current physical activity, h/wk | 2.3±1.0 | 13.2±4.0 | 13.4±3.6 | 1.2±1.7 | 2.16±1.8 (18.8±7.1)‡ | 2.53±1.8 (12.2±1.9)‡ |
| Training experience, y | . . . | 11.4±7.2 | 8.4±3.0 | . . . | 14.9±3.4§ | 14.1±3.5§ |

Data are given as mean±SD. The symbol "*" marks the significant differences between young and older men within the subgroups ie, control, endurance and sprint (*$P$<0.001, Tukey's post hoc test), whereas the symbol "†" marks the significant effects of performed training (sprint or endurance) in respect to age-matched control group (†$P$<0.001,Tukey's post hoc test). BMI indicates body mass index; Y-Con, young control group; Y- End, young endurance trained athletes; Y-Sp, young sprinters; O-Con, older control group; O-End, older endurance trained athletes; O-Sp, older sprinters. Additionally, in the case of older distance runners (O-End) and sprinters (O-Sp), data concerning physical activity level in hours per week (‡) and the training experience in years (§) during the period of their professional training are given.

analysis was performed in each subject, including morphological, biochemical, and endothelial marker measurements. Moreover, a noninvasive assessment of endothelial function and arterial stiffness was conducted (see below).

## Ethical Approval

The study was conducted with the permission of the Local Ethical Committee (No. 48/KBL/OIL/2009), according to the principles established in the Declaration of Helsinki for research on human subjects. All volunteers provided written consent for participation in the study after becoming acquainted with the procedures and purpose of the study.

## Anthropometric Measurements

Anthropometric measurements were performed in the morning, around 15 minutes before blood sampling. Body mass and height of each subject were measured using standard procedures and equipment (Radwag WPT 150; Radwag Wagi Elektroniczne, Radom, Poland), and body fat percentage was determined with a bioelectrical impedance analyzer (UM-018; TANITA Europe GmbH, Sindelfingen, Germany).

## Noninvasive Assessment of Endothelial Function and Arterial Wall Stiffness

On the vascular testing day, subjects were instructed to report to the laboratory between 7:30 and 10:00 AM after 12 hours fasting and abstain from caffeine, vitamin supplements, and exercise. Tests were performed in the supine position in a quiet, semidarkened, and temperature-controlled (22–25°C) room after a 20-minute rest period to obtain a hemodynamically steady state. Heart rate was monitored continuously using a 3-lead ECG. A 14×50 cm automatic cuff (Vendys; Endothelix, Inc., Palo Alto, CA) was placed around the right forearm.

### *Flow-mediated dilation*

Endothelial function was assessed using FMD of the brachial artery. According to the guidelines, the brachial artery was imaged above the antecubital fossa in the longitudinal plane, whereas the occlusion cuff was placed on the forearm.[12] The brachial artery was imaged using a 14-MHz linear-array transducer (Acuson S2000; Siemens, Erlangen, Germany). At baseline, time-averaged velocity was measured and 10-second videos were recorded in order to measure the arterial diameter. A pressure cuff was placed distal to the imaged area and inflated 40 mm Hg above the patients' systolic pressure for 5 minutes. Immediately after releasing the cuff, maximal blood velocity was measured, and 15 seconds after the release, a 120-second video of brachial artery dilation in B-mode was recorded.

Results were obtained by means of a wall-tracking computer system developed in the JCET laboratory.[13] Maximal diameter was obtained with a semiautomatic technique, operating on the basis of the 2 regions of interest, marked by the operator on the first frame of the ultrasound video. The algorithm for tracking the borders of the arterial walls was based on the active contour method.[14] It worked independently for each wall, starting with the initial borders, and marked by the operator.

### *Central blood pressure, PWV, pulse wave analysis, and AI*

cSBP and central diastolic blood pressure were measured noninvasively using the SphygmoCor system (AtCor Medical Pty Ltd, West Ryde, Australia). cPP was calculated as a difference between central systolic and diastolic pressure. Moreover, using this system, PWV was determined at the carotid and radial arterial sites.[15] Pulse wave analysis was performed with the calculation of arterial stiffness parameters

(AI). The SphygmoCor Pulse Wave Velocity Assessment (PWV) system uses applanation tonometry in conjunction with a 3-lead ECG to take sequential measurements at 2 arterial sites. Timing of the onset of systole of the pressure waves was compared with the timing of the corresponding R waves on the ECG recording, with the time delay calculated by the software. The distance from the suprasternal notch to the carotid artery site and the distance from the suprasternal notch to the radial artery site were measured in meters by using a standard measuring tape. The pressure-sensitive transducer (tonometer) was applied to the carotid and radial artery sites along with 3 ECG leads attached to the subjects' chest in order to measure the transit time of the pulse wave from the left ventricle to the carotid artery (t1) and from the left ventricle to the radial artery (t2), respectively. Carotid-radial PWV in individual measurements was calculated as the distance difference between the radial and carotid divided by the difference between t1 and t2 and was used as a measure of peripheral arterial stiffness.

Pulse wave analysis was performed based on the pulse detection at the radial artery with an applanation tonometry sensor using the SphygmoCor system pulse. Aortic pulse from radial pulse was estimated noninvasively using a mathematical model.[16,17] When aortic pulse was obtained, the shape of the aortic pressure pulse was analyzed and calculations were performed to obtain AI, defined as a percentage of the cPP attributed to the reflected pulse wave.[18]

### *Stiffness index*

The SI, a noninvasive indirect technique of measuring arterial stiffness peripherally, was measured by infrared photoplethysmography using the PulseTrace apparatus (CareFusion, Basingstoke, UK). The measurement was based on digital volume pulse measurements, given that the timing of the reflected wave (diastolic component) relative to the direct wave (systolic component) of the pulse wave depends on large artery stiffness. The peak-to-peak time of systolic and diastolic components of the pulse wave is an estimation of the propagation time of the reflected wave. Assuming that the arterial tree length is proportional to the subject body height, the SI was defined as the subject body height divided by the peak (systolic) to peak (diastolic) time. SI=Subject body height/dt where dt is peak to peak time.

### *Carotid intima-media thickness*

cIMT was measured by ultrasound scan (Acuson S2000; Siemens, Erlangen, Germany). cIMT measurements were obtained with the patient lying in the supine position. First, a transverse scan of the common carotid artery was performed from the base of the neck to the carotid bulb to identify a possible focal intima-media thickness thickening and atherosclerotic plaques. Next, each side common carotid artery image from longitudinal postero- and anterolateral scans were obtained to measure intima-media thickness. cIMT was measured in the near and far walls in the most thickened area of each vessel. At least 2 cIMT scans were obtained for each near and far wall. Optimal B-mode settings of gain, depth, focal zone placement, and compression were individually adjusted for each vessel to enhance arterial wall structures and image quality. cIMT was measured by the syngo Arterial Health Package software program, which provides a method for quantifying cIMT. The Arterial Health Package program uses semiautomated border detection to determine the maximum and average thickness of the intima-medial layer of the carotid artery.

## Blood Sampling

Blood samples from the antecubital vein were taken at rest in the fasting state between 7:30 and 9:00 AM. Blood for parameters analyzed in plasma (pl), that is, tumor necrosis factor-α ($[TNF\text{-}\alpha]_{pl}$), interleukin-6 ($[IL\text{-}6]_{pl}$), nitrite ($[NO_2^-]_{pl}$), nitrate ($[NO_3^-]_{pl}$), and prostacyclin metabolite ($[6\text{-keto-}PGF_{1\alpha}]_{pl}$), was collected in plain tubes containing EDTA and then centrifuged at 653*g* for 15 minutes at 4°C.

Blood for parameters analyzed in serum $_{(s)}$, that is, glucose ($[Glu]_s$), lipid profile, prealbumin ($[Prealbumin]_s$), C-reactive protein ($[CRP]_s$), α-acid glycoprotein ($[AAG]_s$), insulin ($[INS]_s$), and hyaluronic acid ($[HA]_s$) concentrations, was collected in plain tubes with a clotting activator and left to clot for a minimum of 30 minutes at room temperature. Then, samples were centrifuged at 1469*g* for 10 minutes at 4°C. Plasma and serum were stored at −80°C until analysis.

## Blood Parameter Analysis

Serum glucose ($[Glu]_s$), total serum cholesterol ($[TC]_s$), triacylglycerol ($[TG]_s$), high-density lipoprotein cholesterol ($[HDL]_s$), and low-density lipoprotein cholesterol ($[LDL]_s$) concentrations were measured on a Cobas c501 analyzer (Roche Diagnostics, Mannheim, Germany), using an enzyme hexokinase method ($[Glu]_s$) and an enzymatic colorimetric method ($[TC]_s$, $[TG]_s$, $[HDL]_s$, $[LDL]_s$). $[CRP]_s$, $[AAG]_s$ and $[Prealbumin]_s$, were determined by immunonephelometry on a BN-ProSpec analyzer (Siemens, Marburg, Germany) and ferritin by the chemiluminescence method using the Architect i1000SR analyzer (Abbott Laboratories, Chicago, IL).

Insulin concentration was measured by electrochemiluminescence immunoassay using a Cobas e411 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Detection limits were 0.8 nmol/L. The intra- and interassay coefficients of variation for this measurement were <2.1% and 2.9%, respectively.

Interleukin 6 ($[IL\text{-}6]_{pl}$) concentration was determined by ELISA, according to the directions provided by the kit

manufacturers (R&D Systems, Inc., Minneapolis, MN). The detection limit was 0.039 ng/L. The intra- and interassay coefficients of variation for this measurement were <8% and 10%, respectively.

Estimation of plasma nitrite ($[NO_2^-]_{pl}$), nitrate ($[NO_3^-]_{pl}$), $[TNF\text{-}\alpha]_{pl}$, $[6\text{-keto-}PGF_{1\alpha}]_{pl}$, and serum hyaluronic acid ($[HA]_s$) concentrations were evaluated as previously described.[19,20]

Moreover, the homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as follows: HOMA-IR = [Insulin (μU/mL)×Glucose (mmol/L)]/22.5.

## Statistical Analysis

The results obtained in this study are presented as means, SDs, and 95% CIs. The data points that deviated from the group means by more than 3 SDs were treated as outliers and excluded from further analysis.

In order to quantify the dependence of noninvasive parameters of arterial stiffness and endothelial function (FMD) on body composition in the studied group of subjects (n=94), Spearman's rank-correlation coefficient was used because of the non-normal pooled data distributions.

In order to analyze the impact of "age" and "training in youth" on the presented variables, the data were analyzed using 2-way ANOVA with a post hoc Tukey test performed to identify significant differences between groups. Normality of distribution was tested with the Shapiro-Wilk test. Homogeneity of variance was tested using the Levene test. No serious violations of the assumptions were detected (the fraction of rejected null hypotheses in this multiple testing was roughly of the order of the assumed significance level).

The size of the data set was limited by the number of former professional athletes available for the study. In order to estimate the sensitivity of the experiment, some ANOVA power calculations were performed. For the balanced 2×3 ANOVA design with total number of 90 observations, and for the standard effect sizes f=0.1, 0.25, 0.40, conventionally attributed to small, medium, and large effects, the powers of the corresponding F-tests were 0.15, 0.65, and 0.96 for the 2-levels age effect, and 0.12, 0.53, and 0.92 for the 3-levels training effect and for the interaction.

In the case of a few variables (PWV, $[AAG]_s$, $[IL\text{-}6]_{pl}$, and $[NO_2^-]_{pl}$), when significant interactions between age and training were found in 2-way ANOVA, a second-stage analysis was performed with two 1-way ANOVA models with age-matched control groups, fitted separately in the group of young athletes (which were active at the time of measurements) and in the group of older former athletes (who finished their sport career ~27 years before this study and did not continued their training). The significant effects in ANOVA are presented as least squares means and standard errors. The significance of the differences with respect to control groups was checked using Dunnett's post hoc test with 2-sided *P* values. Dunnett's test is usually more powerful than Tukey's test, because of a smaller number of comparisons performed. Hence, whenever the main interest is in comparisons to a single natural reference group, Dunnett's test is usually preferred. This is the case in our separate studies of young (Y) and older (O) groups in cases with significant interactions detected in 2-way ANOVA. On the other hand, with 2-way ANOVA, there is no single reference group and Dunnett's test could only be used separately for the 2 factors. It is then more natural, however, to use Tukey's test, and we did so. Significance was set at $P<0.05$. The analysis was performed with the statistical package, STATISTICA (version 13.1; StatSoft, Tulsa, OK).

# Results

## Characteristics of the Studied Group of Men

Anthropometric data, basic blood parameters, and physical training background characteristics for the studied men are given in Table 1. The body composition of older men (60.0±6.9 years) was characterized by a significantly ($P<0.05$) higher body mass, body mass index (BMI), and body fat, when compared with young men (23.9±5.3 years; Table 1).

The body mass of sprinters (young and older) was significantly higher than in age-matched controls (Table 1) and higher ($P=0.006$, Tukey's post hoc test) than in endurance-trained men (young and older). No effect of performed training on BMI and body fat was reported (Table 1).

## The Impact of Aging and Professional Physical Training on the Arterial Wall Stiffness Indices and Central Pressure in the Studied Group of Men

Older men were characterized by significantly higher central AI (−4.7±1.17 versus 27.2±1.17%, respectively, for Y and O group; $P<10^{-4}$; Figure 1A) and SI (6.6±0.33 versus 11.4±0.32 m/sec, respectively, for Y and O group; $P<10^{-4}$; Figure 1C) when compared with young men. An increase in PWV with aging was influenced by performed training, as observed from the significant interaction in the model for PWV ($P=0.03$; Figure 1B). Endurance training at a young age increases PWV (7.25±0.18 versus 7.78±0.19 m/sec, respectively, for the Y-Con and Y-End group). However, after training cessation, this effect seems to disappear and only the general age-related increase was observed (7.48±0.13 versus 8.88±0.13 m/sec, respectively, for the Y and O group). Moreover, higher cSBP (108±1.4 versus 130±1.4 mm Hg, respectively, for Y and O men; $P<10^{-4}$; Figure 1D), higher diastolic blood pressure (71±1.1 versus 82±1.1 mm Hg, respectively, for Y and O men; $P<10^{-4}$), and higher cPP

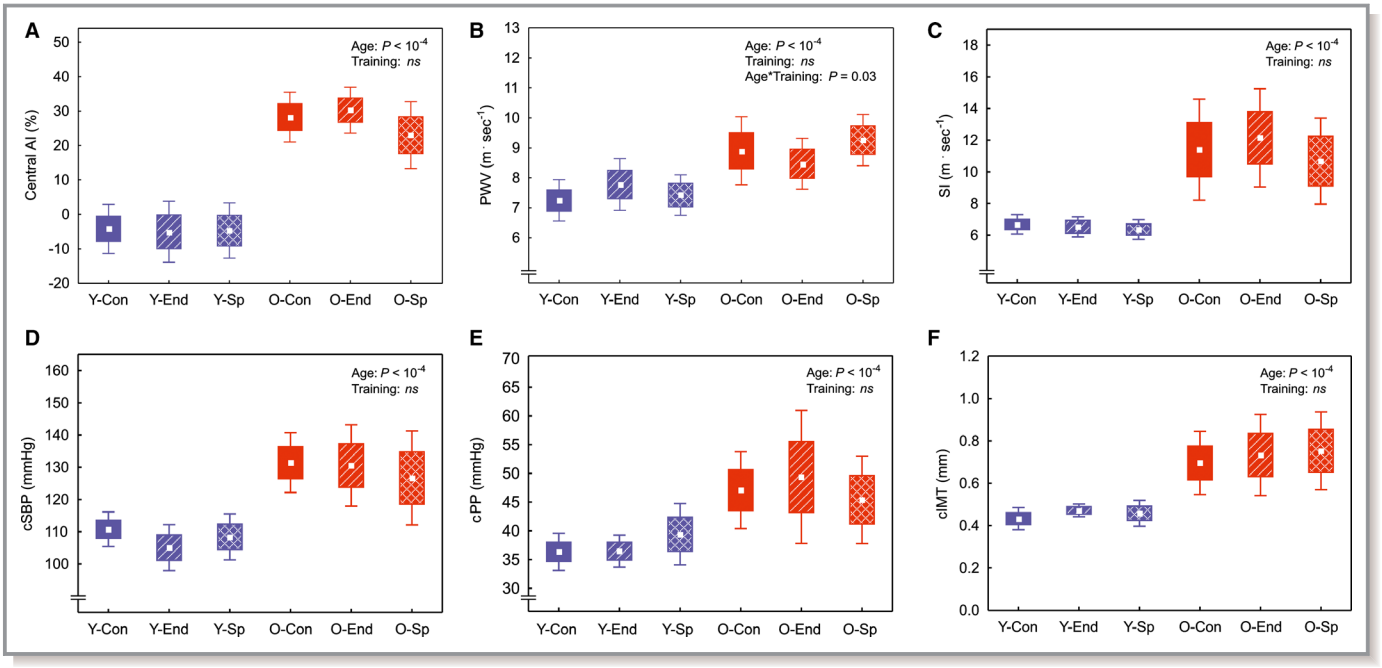


**Figure 1.** The impact of aging and the professional physical training (endurance or sprint) performed at young age on the arterial stiffness parameters, central arterial pressures, and carotid-intima media thickness in the studied group (n=94). Central augmentation index (central AI; **A)**; pulse wave velocity (PWV; **B)**; stiffness index (SI; **C)**, central systolic blood pressure (cSBP; **D)**; central pulse pressure (cPP; **E)**; and carotid intima-media thickness (cIMT; **F)**. Y-Con, young control group; Y-End, young endurance trained athletes; Y-Sp, young sprinters; O-Con, older control group; O-End, older endurance trained athletes; O-Sp, older sprinters. Boxes and whiskers represent, correspondingly, the 95% CIs for means and SDs. The effect of aging is shown by different colors of boxes and whiskers (blue for young and red for older groups of subjects), and the effect of training is marked by different box templates (striped pattern for endurance trained athletes and checkered pattern for sprinters). The impact of factors: Age and Training on the analyzed variables is presented (2-way ANOVA, 2-sided *P* value). In case of PWV, significant interaction between the factors was detected (2-way ANOVA, Age*Training).

(37.4±1.0 versus 47.3±1.0 mm Hg, respectively, for Y and O men; $P<10^{-4}$; Figure 1E) has been found in older men when compared with young men. In addition, a significantly greater ($P<10^{-4}$) cIMT was present in older men (0.73±0.02 mm) compared with young men (0.45±0.02 mm; Figure 1F). No effect of the type of training performed (endurance or sprint; $P>0.05$) on central AI, SI, cSBP, central diastolic blood pressure, and cPP and on cIMT was found in the group of studied men (Figure 1A, 1C through 1F).

## The Impact of Aging and Professional Physical Training on the Endothelial Function and Endothelial Glycocalyx Layer Integrity in the Studied Group of Men

Endothelial function assessed by FMD was significantly attenuated in older men when compared with young men (5.46±0.37 versus 4.11±0.36%, respectively, for Y and O men; $P=0.01$; Figure 2A). In addition, a significant decrease in plasma 6-keto-$PGF_{1\alpha}$ (7202±370 versus 4959±357 pg/mL, respectively, for Y and O men; $P<10^{-4}$; Figure 2D) and plasma nitrate concentration (30.43±1.50 versus 19.33±1.53 µmol/L, respectively, for Y and O men; $P<10^{-4}$; Figure 2C) with aging was found. A significant age-dependent increase in serum hyaluronic acid concentration $[HA]_s$—an indirect marker of glycocalyx layer integrity—was observed (32.6±4.21 versus 65.6±4.21 ng/mL, respectively, for Y and O men; $P<10^{-4}$; Figure 1E). No effect of training on FMD, 6-keto-$PGF_{1\alpha}$, nitrate, and hyaluronic acid concentration was present ($P>0.05$; Figure 2A, 2C through 2E). Plasma nitrite concentration increased with age in the control group (0.29±0.03 versus 0.45±0.04 µmol/L, respectively, for Y and O men; $P=0.01$), whereas in former sprinters and endurance trained athletes, it remained unchanged (Figure 2B).

## The Impact of Aging and Professional Physical Training on the Blood Lipid Profile and Glucose Homeostasis

Age-dependent changes in blood lipid profile in the studied group were characterized by significant increase in total cholesterol (4.34±0.14 versus 5.44±0.14 mmol/L, respectively, for Y and O men; $P<10^{-4}$; Figure 3A), triacylglycerol concentration (1.01±0.07 versus 1.29±0.07 mmol/L, respectively, for Y and O men; $P=0.008$; Figure 3B), LDL-cholesterol concentrations (2.55±0.13 versus

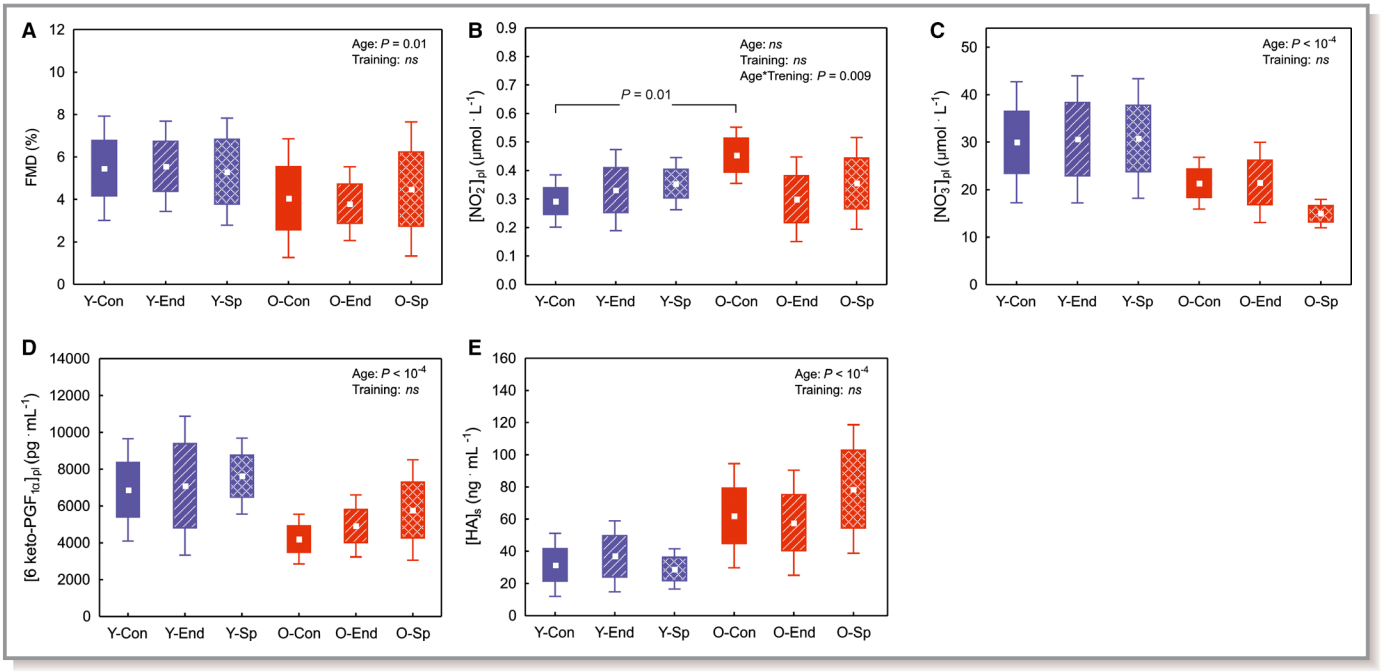


**Figure 2.** The impact of aging and the professional physical training (endurance or sprint) performed at young age on the endothelial function and glycocalyx layer integrity in the studied group of men (n=94). Flow-mediated dilation (FMD; **A)**; plasma nitrite concentration ($[NO_2^-]_{pl}$; **B)**, plasma nitrate concentration ($[NO_3^-]_{pl}$; **C)**; plasma 6-keto-$PGF_{1\alpha}$ ($[6\text{-keto-}PGF_{1\alpha}]_{pl}$; **D)**, and serum hyaluronic acid concentration ($[HA]_s$; **E)**. Y-Con, young control group; Y-End, young endurance trained athletes; Y-Sp, young sprinters; O-Con, older control group; O-End, older endurance trained athletes; O-Sp, older sprinters. Boxes and whiskers represent, correspondingly, the 95% CIs for means and SDs. The effect of aging is shown by different colors of boxes and whiskers (blue for young and red for older groups of subjects), and the effect of training is marked by different box templates (striped pattern for endurance trained athletes and checkered pattern for sprinters). The impact of factors: Age and Training on the analyzed variables is presented (2-way ANOVA, 2-sided *P* value). In the case of $[NO_2^-]_{pl}$, a significant difference between Y-Con and O-Con is shown (Tukey's post hoc test).



3.62±0.13 mmol/L, respectively, for Y and O men; $P<10^{-4}$; Figure 3D) whereas no changes in HDL-cholesterol concentration with aging were observed (1.48±0.05 versus 1.42±0.05 mmol/L, respectively, for Y and O men; Figure 3C). No impact of performed training on the lipid profile was observed, except a clear tendency ($P$=0.07) to higher HDL-cholesterol concentration in endurance-trained athletes when compared with age-matched controls (1.60±0.06 versus 1.41±0.06 mmol/L, respectively, for the End and Con group; Figure 3C). Moreover, endurance-trained athletes revealed a significantly higher HDL-cholesterol concentration (1.60±0.06 versus 1.35±0.06 mmol/L, respectively, for endurance-trained athletes and sprinters; $P$=0.01; Figure 3C) than sprinters of the same age.

Aging was accompanied by a significant increase in basal blood glucose concentration (4.95±0.08 versus 5.49±0.08 mmol/L, respectively, for Y and O men; $P<10^{-4}$; Figure 3E) and an enhancement of HOMA-IR (1.22±0.12 versus 1.69±0.12, respectively, for Y and O men; $P$=0.005; Figure 3F). No effect of training ($P$>0.05) on blood glucose concentration was observed (Figure 3E). A clear tendency ($P$=0.06, Tukey's post hoc) to lower HOMA-IR has been observed in the groups of endurance-trained young and older athletes when compared with age-matched controls (1.17±0.15 versus 1.66±0.14, respectively, for the End and Con group; Figure 3F).

## The Impact of Aging and Professional Physical Training on Blood Inflammatory Markers

Aging significantly increased blood inflammatory markers such as serum C-reactive protein (0.359±0.143 versus 1.345±0.139 g/L, respectively, for Y and O men; $P<10^{-4}$; Figure 4A), serum prealbumin (0.269±0.008 versus 0.298±0.008 g/L, respectively, for Y and O men, $P$=0.01; Figure 4B), plasma TNF-α (1.16±0.06 versus 1.51±0.06 pg/mL, respectively, for Y and O men; $P$=0.0001; Figure 4D), and serum ferritin concentration (64±9.0 versus 144±9.1 ng/mL, respectively, for Y and O men; $P<10^{-4}$; Figure 4F). No impact ($P$>0.05) of performed training on the above-mentioned inflammatory markers was observed.

In the case of α-1-acid glycoprotein (AAG) and interleukin-6 concentrations, training performed at a young age modified the age-dependent changes in these inflammatory markers. Endurance training at a young age decreases AAG

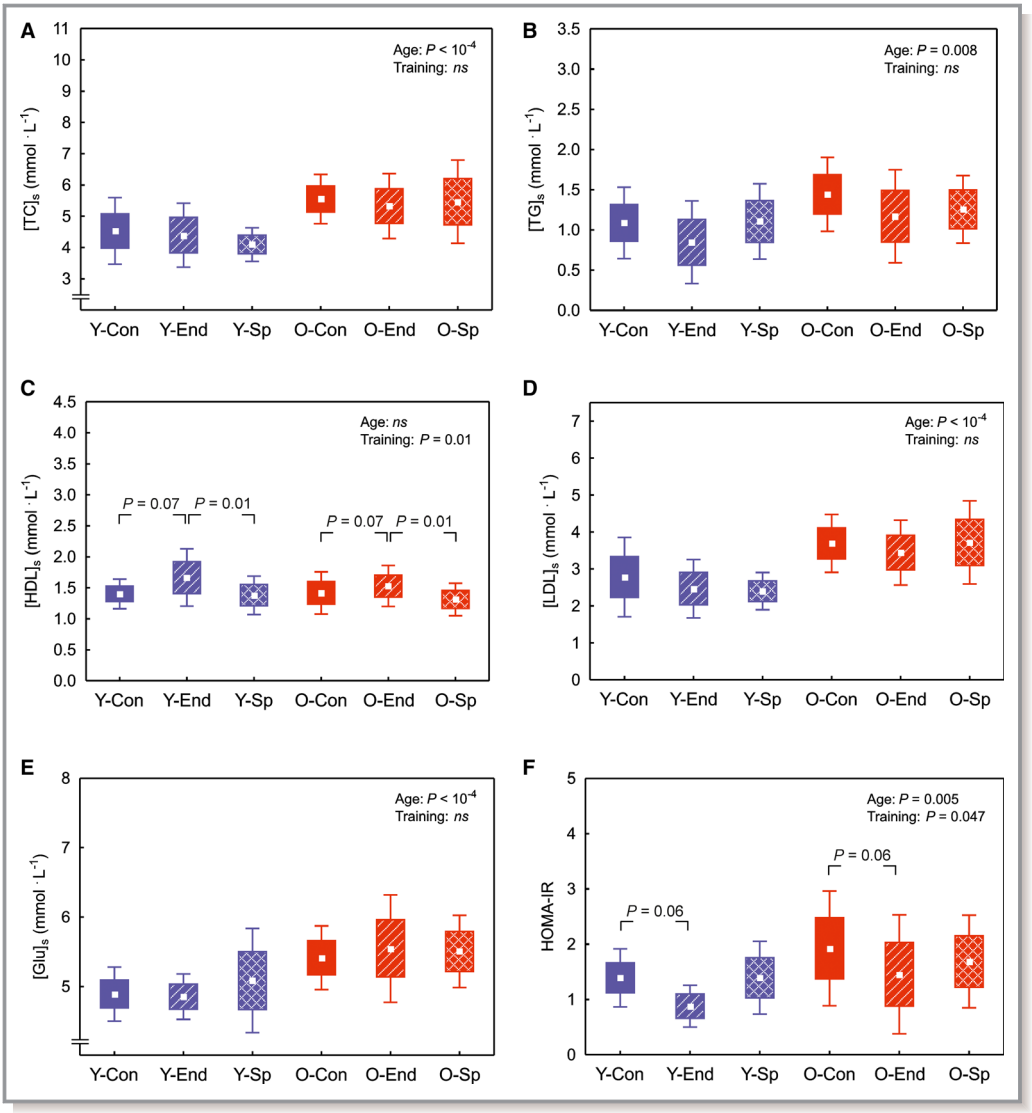


**Figure 3.** The impact of aging and the professional physical training (endurance or sprint) performed at young age on the blood lipid profile and glucose homeostasis in the studied group of men (n=94). Serum total cholesterol concentration ($[TC]_s$; **A)**; serum triacylglycerol concentration ($[TG]_s$; **B)**; serum high-density lipoprotein cholesterol concentration ($[HDL]_s$; **C)**; serum low-density lipoprotein cholesterol concentration ($[LDL]_s$; **D)**; serum glucose concentration ($[Glu]_s$; **E)**; and the homeostatic model assessment of insulin resistance (HOMA-IR; **F)**. Y-Con, young control group; Y-End, young endurance trained athletes; Y-Sp, young sprinters; O-Con, older control group; O-End, older endurance trained athletes; O-Sp, older sprinters. Boxes and whiskers represent, correspondingly, the 95% CIs for means and SDs. The effect of aging is shown by different colors of boxes and whiskers (blue for young and red for older groups of subjects), and the effect of training is marked by different box templates (striped pattern for endurance trained athletes and checkered pattern for sprinters). The impact of factors: Age and Training on the analyzed variables is presented (2-way ANOVA, 2-sided *P* value). In the case of $[HDL]_s$ and HOMA-IR, the *P* value denoted a difference between endurance-trained athletes and age-matched controls as well as between endurance-trained athletes and sprinters of the same age (Tukey's post hoc test).

(0.681±0.031 versus 0.562±0.033 g/L, respectively, for the Y-Con and Y-End group), but this effect seems to disappear after training cessation and only the increase with age is observed (0.64±0.02 versus 1.73±0.02 g/L, respectively, for Y and O men; *P*=0.02; Figure 4C). Moreover, $[AAG]_s$ in endurance-trained young athletes is significantly lower than in the age-matched control group (*P*=0.02, Dunnett's test), whereas no effect of training performed in the past (endurance or sprint) on $[AAG]_s$ is present in former athletes when compared with age-matched controls (Figure 4C). Professional training performed at a young age (endurance and sprint) attenuates $[IL\text{-}6]_{pl}$ concentration, but this effect of

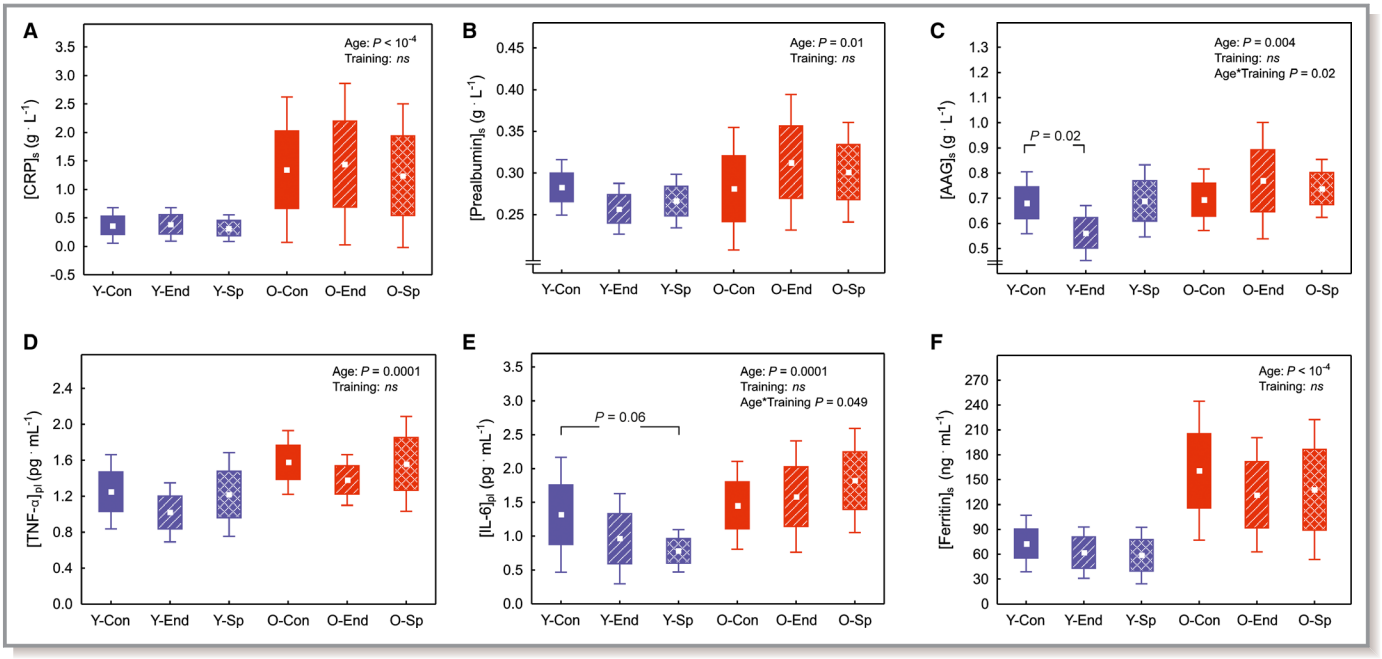


**Figure 4.** The impact of aging and the professional physical training (endurance or sprint) performed at young age on the inflammatory markers in the studied group of men (n=94). Serum C-reactive protein concentration ($[CRP]_s$; **A)**; serum prealbumin concentration ($[Prealbumin]_s$; **B)**; serum α-1-acid glycoprotein concentration ($[AAG]_s$; **C)**; plasma tumor necrosis factor α concentration ($[TNF\text{-}\alpha]_{pl}$; **D)**; plasma interleukin-6 concentration ($[IL\text{-}6]_{pl}$; **E)**; serum ferritin concentration ($[Ferritin]_s$; **F)**. Y-Con, young control group; Y-End, young endurance-trained athletes; Y-Sp, young sprinters; O-Con, older control group; O-End, older endurance-trained athletes; O-Sp, older sprinters. Boxes and whiskers represent, correspondingly, the 95% CIs for means and SDs. The effect of aging is shown by different colors of boxes and whiskers (blue for young and red for older groups of subjects), and the effect of training is marked by different box templates (striped pattern for endurance trained athletes and checkered pattern for sprinters). The impact of factors: Age and Training on the analyzed variables is presented (2-way ANOVA, 2-sided *P* value). In the case of $[AAG]_s$ and $[IL\text{-}6]_{pl}$, a significant interaction between the factors was detected (2-way ANOVA, Age*Training). Moreover, in the case of $[AAG]_s$, a significant difference between young endurance-trained athletes and age-matched controls is presented, whereas in the case of $[IL\text{-}6]_{pl}$, a significant difference is shown between young sprinters and age-matched controls (Dunnett's test).

training disappeared with aging, as manifested by the significant interaction ($P$=0.049; Figure 4E). A clear tendency to lower $[IL\text{-}6]_{pl}$ in young sprinters when compared with the age-matched control group was observed (1.32±0.16 versus 1.46±0.19 pg/mL, respectively, for Y and O men; $P$=0.06; Dunnett's test), whereas no effect of training performed at a young age (sprint or endurance) was found in former athletes on $[IL\text{-}6]_{pl}$ (Figure 4E).

## Correlation Analysis

Based on the data showing that excess of fat or dysfunctional fat tissue is involved in age-related metabolic dysfunction and accelerates the onset of age-related diseases, including cardiovascular disease,[21] we have performed the correlation analysis between body composition and cardiovascular risk factors.

In the studied group of men (n=94), body fat indices, that is, body fat percentage and BMI, correlated positively ($P<10^{-4}$) with the parameters of central and peripheral arterial stiffness (central AI, PWV, and SI), central pressure (cSBP, cPP), as well as with a marker of atherosclerosis—cIMT. Moreover, body fat and BMI correlated positively with blood glucose and insulin resistance as well as with total cholesterol and LDL-cholesterol (Table 2).

In addition, we found that BMI and body fat percentage correlated positively with inflammatory markers, including a major proinflammatory cytokine—TNF-α as well as with a marker of inflammation and oxidative stress—ferritin (Table 2).

## Discussion

The main finding of the present study is that professional physical training (endurance or sprint) performed at a young age by athletes competing at a national/international level did not modify the age-dependent decline of endothelial function and arterial stiffness with aging.

## The Impact of Aging on Arterial Stiffness and Endothelial Function

Age-dependent arterial stiffening and endothelial dysfunction are recognized as strong, independent predictors of CVD,

**Table 2.** Spearman Correlations of Body Fat Indices With Arterial Wall Stiffness and Endothelial Function Parameters As Well As With Metabolic Profile and Blood Inflammatory Markers in the Group of Men Aged 18 to 75 Years (n=94).

| | BMI, kg/m$^2$ | Body Fat, % |
|---|---|---|
| Central AI, % | 0.54‡ | 0.74‡ |
| PWV, m/sec | 0.49‡ | 0.59‡ |
| SI, m/sec | 0.48‡ | 0.70‡ |
| Central SBP, mm Hg | 0.60‡ | 0.75‡ |
| Central PP, mm Hg | 0.45‡ | 0.54‡ |
| cIMT, mm | 0.53‡ | 0.67‡ |
| FMD, % | −0.05 | −0.14 |
| $[NO_2^-]_{pl}$, μmol/L | 0.16 | 0.09 |
| $[6\text{-keto-PGF}_{1\alpha}]_{pl}$, pg/mL | −0.24* | −0.37† |
| $[HA]_s$, ng/mL | 0.30‡ | 0.40‡ |
| $[Glu]_s$, mmol/L | 0.42‡ | 0.55‡ |
| HOMA-IR | 0.48‡ | 0.47‡ |
| $[TC]_s$, mmol/L | 0.44* | 0.57‡ |
| $[HDL]_s$, mmol/L | −0.22* | −0.14 |
| $[LDL]_s$, mmol/L | 0.47‡ | 0.60‡ |
| $[CRP]_s$, mg/L | 0.55‡ | 0.66‡ |
| $[AAG]_s$, g/L | 0.39‡ | 0.40‡ |
| $[IL\text{-}6]_{pl}$, pg/mL | 0.42‡ | 0.47‡ |
| $[TNF\text{-}\alpha]_{pl}$, pg/mL | 0.27† | 0.37‡ |
| $[Ferritin]_s$, ng/mL | 0.48‡ | 0.60‡ |

The symbols represent the significance of the given correlation (*$P$<0.05; †$P$<0.01; ‡$P$<0.001). $[NO_2^-]_{pl}$ indicates plasma nitrite concentration; $[6\text{-keto-PGF}_{1\alpha}]_{pl}$, plasma 6-keto-$PGF_{1\alpha}$; $[AAG]_s$, serum α-1-acid glycoprotein concentration; $[CRP]_s$, serum C-reactive protein; $[Ferritin]_s$, serum ferritin concentration; $[Glu]_s$, serum glucose concentration; $[HA]_s$, serum hyaluronan concentration; $[HDL]_s$, serum high-density lipoprotein cholesterol concentration; $[IL\text{-}6]_{pl}$, plasma interleukin-6 concentration; $[LDL]_s$, serum low-density lipoprotein cholesterol concentration; $[TC]_s$, serum total cholesterol concentration; $[TNF\text{-}\alpha]_{pl}$, plasma tumor necrosis factor-α concentration; BMI, body mass index; central AI, central augmentation index; central PP, central pulse pressure; central SBP, central systolic blood pressure; cIMT, carotid-intima media thickness; FMD, brachial flow-mediated dilation; HOMA-IR, homeostatic model assessment of insulin resistance; PWV, pulse wave velocity; SI, stiffness index.

affecting each other and accelerating in this way vascular dysfunction.[22]

The background of an arterial stiffness is rather complex and can occur as a result of changes in the vascular matrix (an increase in collagen and its cross-linking accompanied by a decrease in elastin) or the function of vascular smooth muscle cells.[23] As demonstrated, $NO^•$ bioavailability plays an important role in this process, given that in the physiological condition, $NO^•$ inhibits the cross-linking enzyme, transglutaminase 2 (TG2) activity[24] and a decrease in $NO^•$ leads to an increase in TG2 secretion to the extracellular matrix and its activation.[6,7] TG2, ubiquitously expressed in the vasculature, regulates vascular stiffness not only through catalazing cross-linking of extracellular matrix proteins, but also by changing the behavior of smooth muscle cells (ie, their adhesion, motility, and proliferation)[25]; hence, TG2 activity seems to be an important link between endothelial dysfunction and arterial stiffening.

In the present study, we found, as others did before, that aging is accompanied by a significant increase in arterial stiffness (Figure 1A through 1C), central pressure (Figure 1D through 1E), and cIMT (Figure 1F).[26,27] The age-dependent decrease in brachial FMD (Figure 2A), which reflects the attenuation of $NO^•$-mediated vascular smooth muscle relaxation,[28,29] in addition to a decrease in plasma 6-keto-$PGF_{1\alpha}$—a metabolite of prostacyclin (Figure 2D)—shows that age-dependent endothelial dysfunction accompanies arterial stiffness. When considering the decrease in basal releases of plasma 6-keto-$PGF_{1\alpha}$, a metabolite of prostacyclin with aging (Figure 2D), one should remember that this can increase the risk of cardiac events during exercise[30,31] and might contribute to the decrease in the magnitude of $VO_{2max}$—the key index of humans' exercise capacity, which also declines with aging.[32,33]

Moreover, we found that aging accelerates endothelial glycocalyx shedding, as reflected by a higher serum hyaluronan concentration in older men (Figure 2E), one of the indirect indices of glycocalyx layer integrity. Endothelial glycocalyx, a carbohydrate-rich gel layer localized between blood flow and the endothelial cell surface, is one of the important regulator of endothelial function and is a hallmark of vascular health.[34] An intact glycocalyx is critical to mechanotransduction, vascular permeability, angiogenesis, and protection of endothelial cells from plasma oxidants and inflammatory cytokines.[34] Glycocalyx degradation (by among others oxidative stress and inflammation) inhibits $NO^•$ production in response to shear stress and hence leads to a decrease in $NO^•$ bioavailability and endothelial dysfunction.[35,36] Interestingly, as recently shown, glycocalyx deterioration occurring during primary aging is accompanied by an impairment of microvascular perfusion and precedes age-dependent vascular pathology.[37] Therefore, age-dependent glycocalyx degradation leading to micro- and macrovascular dysfunction is suggested to be an important risk factor in CVD.[38] Our study clearly demonstrates that endothelial glycocalyx shedding with aging (Figure 2E) accompanies endothelial dysfunction (Figure 2A) and arterial stiffening (Figure 1A through 1C), which is in agreement with the data presented by Machin et al.[37]

An attenuated endothelial function (Figure 2A), disturbed glycocalyx integrity (Figure 2E), and an enhanced arterial stiffness (Figure 1A through 1C) in older men compared with younger men were accompanied by an age-dependent increase in blood inflammatory markers, including plasma TNFα, IL-6 (Figure 4D and 4E), and serum ferritin concentration (Figure 4F)—also recognized as an oxidative stress marker.[39] These results support the findings that glycocalyx deterioration, endothelial dysfunction, and an increase in

arterial stiffening with aging is mainly dedicated to 2 mutually driving processes, that is, an age-dependent increase in oxidative stress and chronic low-grade inflammation.[40,41]

The background of an age-dependent increase in oxidative stress and inflammation leading to vascular dysfunction is suggested to be dedicated, among others, to the changes in body composition with aging, specifically to an increase in body fat,[21,42,43] which has been shown to be a major contributor to chronic low-grade inflammation.[21] The proinflammatory cytokine, TNF-α, produced by adipose tissue,[44] is recognized as a key factor involved in the arterial stiffening background, given that it is involved in an increase of TG2 expression[45–47] as well as being implicated in the induction of proinflammatory, procoagulant, and proliferative genes in vasculature.[48] In addition, TNF-α has been shown to increase arginase activity, which, as a consequence, leads to a decrease in $NO^{\bullet}$ bioavailability.[49]

In the present study, an age-dependent increase in body fat percentage, even within the normal range (from 14% in young men to around 25% of body mass in older men; Table 1), might be implicated in an increase in inflammation and oxidative stress (Figure 4A through 4F) with aging, given that adipose tissue becomes dysfunctional with aging.[44] Indeed, as presented, body fat percentage in the whole group of subjects (n=94) correlated positively with inflammatory and oxidative stress markers (Table 2). This result indicates that adipose tissue might be involved in enhanced inflammation and oxidative stress with aging. Finally, the strong, positive correlation between body fat indices (BMI, body fat percentage) and arterial stiffness parameters (Table 2) clearly demonstrates that arterial stiffening, a hallmark of vascular aging,[3] depends on body fat content. This probably results from age-dependent chronic low-grade inflammation (reflected by an increased TNF-α) in which dysfunctional adipose tissue plays an important role.[21] This is in agreement with data presented recently by others.[50,51]

## The Impact of Professional Physical Training (Endurance Versus Sprint) on Arterial Stiffness and Endothelial Function in Young and Older Athletes

It is generally accepted that endurance training of low-to-moderate intensity exerts beneficial effects in the cardiovascular system.[9,52] Even short-term endurance training has been shown to decrease oxidative stress[53] and inflammation,[54] enhance $NO^{\bullet}$ bioavailability,[55] and improve endothelial glycocalyx layer integrity.[56] It needs to be underlined that regular aerobic exercise is suggested as one of the most important healthy lifestyle strategies for optimal cardiovascular aging.[2,3,5,9]

Interestingly, the effects of professional training (endurance or sprint) performed at a young age on cardiovascular risk factors in former athletes is unclear. In general, the analysis of mortality ratio and cause of death in the group of elite athletes when compared with the general population show that elite athletes live longer and possess lower cardiovascular and cancer risks than the general population.[57–59] Moreover, with regard to athletes' life span longevities, some studies show a favorable survival outcome for endurance-trained athletes (eg, long distance runners) when compared with power sport (eg, weightlifters).[57] The recent analysis of the longevity and cause of death (overall deaths) in the group of 2418 (455 died) of French elite athletes, participants of the Olympic Games (from 1912 to 2012), revealed that indeed elite athletes lived ~6.5 years longer than the general population, but it was mainly driven by lower risk of cancer.[59] Interestingly, CVD-related mortality in endurance and precision sports (targeting events) was not significantly different from the general population, whereas athletes from other sports disciplines (including power sports) saved, on average, 1.6 years of life.

In the present study, we found no effect of professional training performed at a young age (which was stopped ≈27 years earlier; see Table 1) on cardiovascular risk factors, that is, central and peripheral arterial stiffness, central blood pressure, cIMT (Figure 1), and brachial FMD (Figure 2A), in former athletes. Moreover, in the former athletes, no effect of the training performed in the past on inflammatory markers (Figure 4A through 4F), LDL-cholesterol (Figure 3D), glucose concentration (Figure 3E), and glycocalyx layer integrity marker, prostacyclin, and $NO^{\bullet}$ metabolites (Figure 2B through 2E) was observed. It is, however, worth noting that professional training performed at a young age was linked with a more preferable inflammatory profile, as judged by lower AAG in young endurance-trained athletes (Figure 4C). However, after training cessation, this effect disappeared and was not present at an older age. Moreover, a clear tendency toward higher HDL-cholesterol and lower insulin resistance assessed by HOMA-IR in young and former endurance-trained athletes when compared with age-matched controls was found (Figure 3C and 3F), which might suggest that endurance training performed at a young age is indeed more preferable for cardiovascular health and insulin sensitivity.[60,61] However, as found in the present study, at the age of 60, it has no impact on the age-related increase in arterial stiffness and the attenuation of endothelial function with aging (Figures 1 and 2).

The lack of a significant impact of training performed at a young age on arterial stiffness and endothelial function might be explained by the long gap from the end of professional training (Table 1). Hence, our results show that the effects of several years of professional training performed at a young age were not generally maintained in older age.

As discussed above, one of the important determinants of arterial stiffness is body fat as the major contributor of chronic low-grade inflammation.[21] In the present study, an

age-dependent increase in body fat (Table 1) and inflammatory markers—including TNF-α (Figure 4D)—was not modified by professional training performed at a young age. This might explain the lack of an impact of professional training performed in the past (≈27 years earlier) on the age-dependent decline in endothelial function (Figure 2A) and increased arterial stiffness (Figure 1A through 1C).

In this study, we also aimed to compare the effect of sprint versus endurance training on the markers of cardiovascular risk in young athletes. Surprisingly, we found no specific effect of sprint versus endurance training on the cardiovascular system at a young age and no delayed effects of sprint versus endurance training on the cardiovascular system (Figures 1 and 2). This provides evidence that the sprint training conducted through sports carriers of professional athletes (for several years; see Table 1) seems to have no harmful effects on the cardiovascular system during carriers as well as in older age, when compared with its status in endurance athletes and in untrained controls.

## Conclusions

In the present study, we found that professional training (endurance or sprint) performed at a young age and regular participation in sports events at a national/international level did not influence the age-dependent decline in endothelial function and an increase in arterial stiffness in older age. Therefore, our results indicate that sports training at a young age, then followed by the low-to-average physical activity level, is not sufficient to slow down the age-related decline in endothelial function and increase in arterial stiffness in men. This result points to age as the major determinant of the status of the arteries, which are not significantly influenced by the history of several years of sports training performed at a young age.

## Study Limitations

One of the limitations of this study is a relatively small amount of subjects within the study groups of athletes (n=15–16 individuals per group). Therefore, our study needs to be expanded in the future to a larger cohort of athletes with varying ages. It would also be interesting to see the comparison of the studied indices of endothelial function and blood variables in the former athletes and controls at a more advanced age (eg, >80 years).

Another limitation to the study is that a couple of older subjects (mainly from the control group, only 1 from former endurance trained and 1 from former sprinters) were on antihypertensive therapy. Further studies on the larger group of subjects are mandatory to elucidate whether antihypertensive therapy in a similar group as in the current study would affect vascular aging. Nevertheless, it is unlikely that possible favorable effects of antihypertensive drugs on vascular wall aging in a control group would change the conclusion of our study.

It should be underlined that our present study involved male subjects only in order to avoid a potential impact of sex differences on the time course of aging-related changes in endothelial function and in arterial stiffness, as reported by others.[62,63] It should be stated that the conclusion of our study cannot be simple extrapolated on the aging female athletes in whom the time course of aging-related changes in endothelial function and in arterial stiffness might be different than in men.[62,63] This is why further studies are needed to establish the impact of aging in former high-class female athletes and women with no history of physical training on the endothelial function and arterial stiffness.

Moreover, further studies are needed to evaluate the relationship between the changes in the $VO_{2peak}$ as an index of cardiorespiratory fitness and the changes in vascular function variables and cardiovascular disease risk parameters in the time course of aging.

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## Author Contributions

Majerczak, Zoladz: study design, performed experiments, data analysis, data interpretation, writing of the manuscript, and final approval of the version to be published; Grandys, Frołow, Zakrzewska: performed experiments, revising manuscript, data interpretation, and final approval of the version to be published; Szkutnik: data analysis, data interpretation, revising manuscript, and final approval of the version to be published; Chlopicki, Niżankowski, Duda: data interpretation, revising manuscript, and final approval of the version to be published.

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## Disclosures

None.

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